Original Article
MicroRNA-561 inhibits gastric cancercell proliferation and invasion by downregulating c-Myc expression

Kun Qian[1], Binglang Mao[2], Wei Zhang[1], Huanwen Chen[3]

Departments of [1]Gastrointestinal Surgery, [3]Cardiothoracic Surgery, [2]The Medical Examination Center, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China, 400016

Abstract: Gastric cancer (GC) causes nearly one million deaths worldwide each year. However, the molecular pathway of GC development remains unclear. Increasing evidences have shown that microRNAs (miRNAs) are highly associated with tumor development. However, relative little is known about the potential role of miRNAs in gastric cancer development. In the present study, we showed that miR-561 was down-regulated frequently in human GCs cell lines and tissues, and its expression was associated with tumor-node-metastasis (pTNM) stage. Enforced expression of miR-561 in GC cells inhibited cell proliferation and invasion in vitro. In contrast, knockdown of miR-561 had the opposite effect on cell proliferation and invasion. Moreover, c-Myc was identified as a potential miR-561 target. Further studies confirmed that miR-561 suppressed the expression of c-Myc by directly binding to its 3'-untranslated region. Restoration of c-Myc in miR-561-overexpressed GC cells reversed the suppressive effects of miR-561 and c-Myc was inversely correlated with miR-561 expression in GC tissues. These results demonstrate that miR-561 acts as a novel tumor suppressor in GC by targeting c-Myc gene and inhibiting GC cells proliferation and invasion. These findings contribute to current understanding of the functions of miR-561 in GC.

Keywords: Gastric cancer, microRNA-561, c-Myc, tumor suppressor

Introduction

Gastric cancer (GC) is the fourth most prevalent cancer worldwide and the second most frequent cause of cancer death, with an estimated 850,000 newly diagnosed cases and 650,000 deaths per year [1, 2]. Despite recent advances in GC therapy, the 5-year overall survival of respectable GC in specialized centers in Europe or the United States wasonly approximately 35% [3, 4]. The 5-year survival rate of metastatic or advanced GC wasnearly 5-20%, with median overall survival only less than 1 year [5]. However, the method for early detection and prediction of lymph node metastasis has not yet been well established. Therefore, it is necessary to identify novel biomarkers toaccurately predict GC development, prognosis, or response to treatment [5, 6].

MicroRNAs (miRNAs) are a new class of endogenous small non-coding regulatory RNAs, which regulate gene expression at the posttranscriptional level [7-9]. MiRNAs play important roles in various biological processes such as cell proliferation, apoptosis, metabolism, and differentiation [2, 10-12]. It is reported that miRNAs are aberrantly expressed in many cancers, affecting cellmigration, invasion, epithelial-to-mesenchymal transition, and metastasis in tumor [13-15]. Some miRNAs act as tumor suppressor genes or oncogenes in human malignancies [16-18]. However, the role of miRNAs in GC remains largely unknown.

Deregulation of miR-561 is a constant event in various cancers, suggesting that miR-561 may play an important role in tumor progression [19, 20]. Yu et al. showed that miR-561 was down-regulated human colon cancer stem cells [21]. However, the expression and function of miR-561 in GC remain uninvestigated. In our study, miR-561 was downregulated in human GC. In addition, miR-561 was associated with lymph node metastasis in an independent GC cohort. Functional investigation indicated that miR-561 might act as a novel antimetastaticmiRNA in GC. Moreover, the c-Myc was identified as a direct and functional target of miR-561.

Materials and methods

Ethics statement

All patients agreed to participate in the study and gave written informed consent. Both this study and consent were approved by the ethical board of the The First Hospital of Chongqing University and complied with the Declaration of Helsinki.

Human samples

Human GCs and their corresponding non-tumorous gastric samples were collected during surgical resection from The First Hospital of Chongqing University from 2008 to 2009. Samples collected were immediately frozen in liquid nitrogen and subsequently stored in -80°C freezer. None of the patients had received radiotherapy or chemotherapy before surgery. The characteristics of the patients are described in Table S1.

Cell lines and cell culture

The following human cell lines were used: SGC-7901, HGC-27, MKN-45, MGC-803, GES and HEK293T. These cell lines were purchased from the Cell Resource Center of the Institute of Basic Medical Sciences at the Chinese Academy of Medical Sciences and the Peking Union Medical College (Beijing, China). Cells were propagated in Dulbecco's modified Eagle's medium (Gibco; Invitrogen; Life Technologies, Germany), which was supplemented with 10% fetal bovine serum (GIBCO, NY, USA), streptomycin (100 μg/ml), and penicillin (100 U/ml).

Plasmids and cell transfection

MiR-561 mimic/inhibitor and controls were purchased from RiboBio (Guangzhou, China). HGC-27 cells were incubated in the six-well plates at 30% confluence one day before transfection. Transfection with miR-561 mimic/inhibitor or controls was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Luciferase assay

The 3'UTRs of human c-Myc mRNAs were amplified by PCR and cloned into pRL-TK to generate the c-Myc reporter. Mutations in these mRNA sequences were prepared using the Quick Change SiteDirected Mutagenesis kit (Stratagene, CA, USA). The plasmid was created using the Plasmid Maxi Kit (Qiagen, CA, USA). Cells were first transfected in 12-well plates using appropriate plasmids. After transfection for 48 hours, cells were harvested and lysed for luciferase assay. Luciferase assays were performed by luciferase assay kit (Promega, Madison, WI). Renilla luciferase was chosen as the normalization.

RNA extraction and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). To measure mRNA expression, quantitative real-time PCR (qRT-PCR) assays were performed using the PrimeScript RT Reagent Kit (TaKaRa) and SYBR Premix Ex Taq (TaKaRa). The expression of c-Myc was detected by Real-Time PCR System real-time PCR System (Applied Biosystems, Carlsbad, USA) with SYBR Premix Ex Taq (Takara, Dalian, China). GAPDH was used as an internal control. The expression of mature miR-561 was detected by TaqMan miRNA assays (Applied Biosystems). U6 small nuclear RNA was used as an internal control (Table S2).

Northern blot analysis

RNAs were electrophoresed on 15% acrylamide and 8 M urea denatures gels, and then transferred onto Hybond N membrane (Amersham Biosciences). The membranes were baked at 80°C for 2 hours. Subsequently, the membranes were hybridized with oligo-nucleotide probes of the miRNAs. The probes of the miRNA-561 were as follows: 5'-CAAAGUUUAAGAUCCUUGAAGU-3'. Probes were 5-end labeled using the polynucleotide kinase in the presence of [r-32P] ATP. Hybridization was done at 39°C in ULTRAhyb™Oligo Hybridization Buffer (Ambion) for 16 hours. The membranes were washed with SSC at 42°C for 3 times and subsequently rehybridized after stripping the oligo nucleotides used as probes in 1% SDS for 30 minutes at 65°C. The U6 RNA was used as an internal control.

Cell proliferation assay

Cells were plated in 96-well plates before transfection. Cells were cultured for 24 h in normal conditions and then transfected with miR-561 mimic or antimiR-561 inhibitor along with paired negative controls. Cell proliferation was
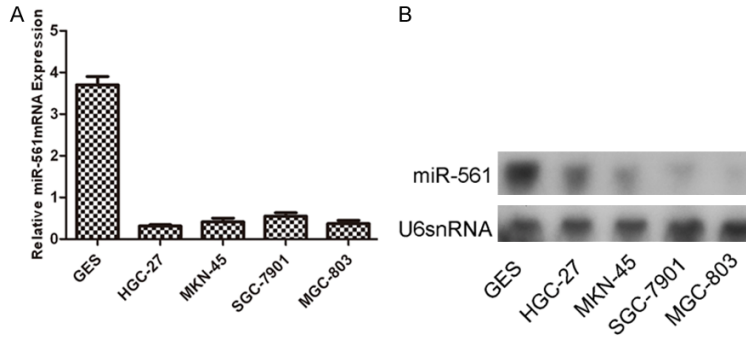
Figure 1. The expression of miR-561 is down-regulated in GC cell lines. A. Down-regulation of miR-561 expression in GC cell lines (SGC-7901, HGC-27, MKN-45, MGC-803) compared with the corresponding controls. Relative expression of miR-561 in four GC cell lines and one normal gastric epithelial-1 cells (GES) was determined by qRT-PCR. U6 snRNA was used as internal control. B. Northern blot analysis of miR-561 expression in four GC cell lines and one normal gastric epithelial-1 cells (GES). U6 was also detected as a loading control.

assessed using Cell Counting Kit 8 assay (Dojindo, Tokyo, Japan) according to manufacturer's protocol.

Invasion assays

Cell invasion was assayed using a transwell chamber (Millipore, USA) with Matrigel (BD, Franklin Lakes, USA). Thetranswell chamber was coated with 30 µl Matrigel, placed into a 24-well plateand incubated for 40 minutes at 37°C. After 48 hours of transfection, cells (8 × 10^4 cells per well) were trypsinized and seeded in chambers, cultured inRPMI 1640 medium containing 2% serum and 600 µl of 10% FBS-1640 in the lower chamber for 24 h. Invasive cells were fixed with 100% methanol for 30 min and non-migrated cells were removed by cotton swabs. Cells on the bottom of the membrane were stained by 0.1% crystal violet (Sigma) for 20 min. Cell images were observed under a phase-contrast microscope (Olympus, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed as follows. Proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% nonfat milk and incubated with the following primary antibodies: rabbit anti-c-Myc monoclonal antibody (1:500; Santa Cruz Biotechnology, USA), mouse anti-GAPDH mAb (1:10,000; Sigma). Enhanced Chemiluminescence (ECL) system (Pierce Biotechnology Inc, USA) was used to detect the bound secondary antibody.

Rescue assays of c-Myc gene expression

The full length of c-MyccDNAs (ORF and 3'UTR) was PCR-amplified and then cloned into the pcDNA 3.1 vector to generate the pcDNA-c-Myc constructs. The primers were used as follows: c-Myc-F (5'-TGCTAAGAAGATTGGTGCTGTA-3') and c-Myc-R (5'-GCGAAG-GGCTGAGACATTTAC). The plasmid was prepared using the Plasmid Maxi Kit (Qiagen, CA, USA). HGC-27 cells were first transfected with miR-561 or a scrambled dsRNAs (60 nM). Cells were cultured for 24 h and thenco-transfected with miR-561 (20 nM) and pcDNA-c-Myc (2.0 µg), miR-561 (20 nM) and pcDNA-empty (2.0 µg). At the indicated time points after hemin addition, cells were harvested and assayed as required.

Statistical analysis

Each experiment was repeated at least three times. Statistical analyses were performed using SPSS 15.0. Data are presented as mean ± standard deviation. Statistical analyses were performed with either an analysis of variance (ANOVA) or Student's t-test. $\alpha = 0.05$ (two-side) was set as the statistical significance level

Results

miR-561 was down-regulated in GC cell lines

As shown in **Figure 1A**, miR-561 was down-regulated in all the GC cell lines compared with GES. Northern blot also showed that the expression level of miR-561 was generally lower in the GC cell lines compared with GES (**Figure 1B**). Thus, we speculated that miR-561 might be a tumor suppressor in GC.

miR-561 expression was down-regulated in both GC tissues

In general, the expression of miR-561 in GC tissues was lower than in adjacent tissues (**Figure 2A**, p<0.001). miR-561 was down-regulated in 44 cases (44/50, 88%) compared with normal adjacent tissues (**Figure 2B**). In addition, the
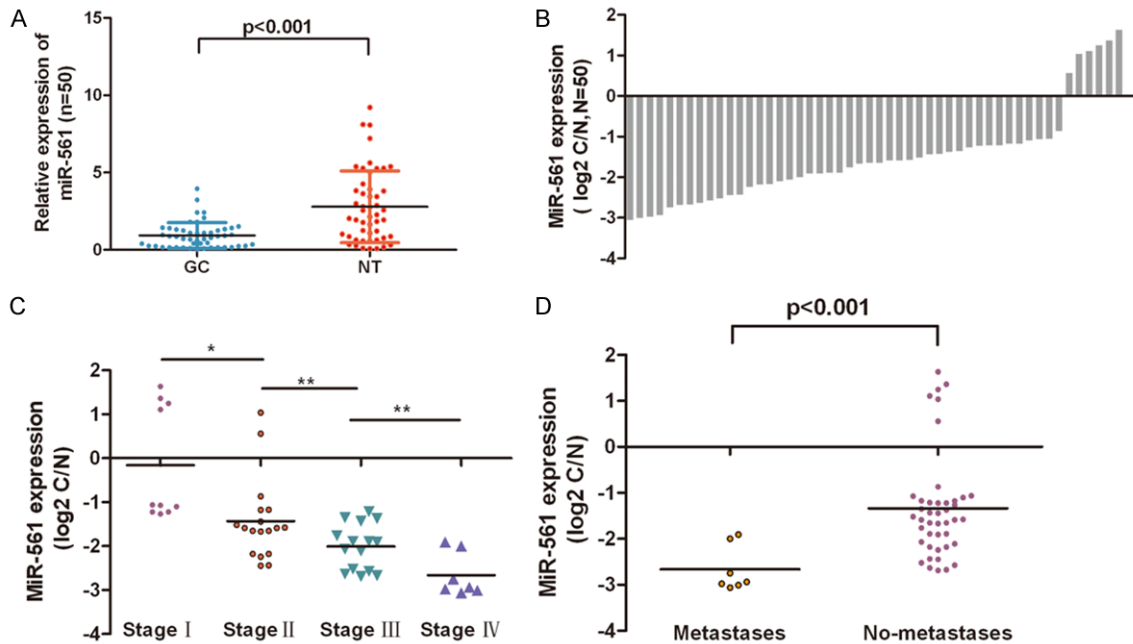
Figure 2. miR-561 expression was down-regulated in both GC tissues. A. Relative miR-561 expression levels in GC tissues and adjacent normal tissues (NT) were determined by qRT-PCR. U6 snRNA was used as internal control. B. miR-561 was detected in 50 pairs of GC tissues and its adjacent normal controls by quantitative RT-PCR. Data are presented as log2 of fold change of GC tissues relative to adjacent normal regions; C and D. The Statistical analysis of the association between miR-561 level and pTNM stage (I, II, III and IV) and pM stage (No metastasis and Metastasis); *p<0.05, and **p<0.01, ***p<0.001.

lower level expression of miR-561 was associated with pTNM stage (**Figure 2C**) and tumor metastasis (p≤0.001, metastasis vs. no metastasis) in GC patients (**Figure 2D**). These data suggested that alterations of miR-561 might be involved in GC progression and metastasis.

miR-561 inhibited GC cell proliferation and invasion

Cells were transfected with scrambled control oligo or miR-561 mimics, control and inhibitor, withhigh transfection efficiency (**Figure 3A** and **3C**). CCK-8 proliferation assay showed that cell proliferation was inhibited in miR-561 mimics-transfected GC cells compared with scrambled oligo-transfected cells or untreated cells in the both HGC-27 cells and MGC-803 cells (**Figure 3B**). Conversely, miR-561 inhibitor increased cell proliferation of the HGC-27 cells and MGC-803 cells (**Figure 3D**). Intriguingly, invasion assay showed that overexpression of miR-561 inhibited the invasion of HGC-27 cells and MGC-803 cells compared with the control whereas miR-561 inhibitor promoted cell invasion (**Figure 3E**).

miR-561 downregulated c-Myc through interaction with its 3'UTR

Bioinformatics analysis using TargetScan showed that c-Myc was a potential target of miR-561 (**Figure 4A**). Real-time RT-PCR and Western blot analysis of c-Myc in HGC-7 cell lines showed that miR-561 mimic transfection inhibited c-Myc mRNA and protein expression (**Figure 4C** and **4D**). The effect of miR-561 on the translation of c-Myc mRNA into protein was assessed by luciferase reporter assay (**Figure 4B**). Overexpression of miR-561 reduced luciferase activity of reporter gene with wild type, but not the mutant c-type, indicating that miR-561 directly targeted c-Myc 3'UTR.

Overexpression of c-Myc partially rescued miR-561-inhibited cell proliferation and invasion

C-Myc expression was restored by cotransfection of a construct expressing c-Myc and miR-561 in HGC-7 cells, as confirmed by Western blot (**Figure 5A**). This restoration of c-Myc increased the proliferation and invasive capabilities of GC cells. More importantly, restoration of c-Myc could reverse the proliferation
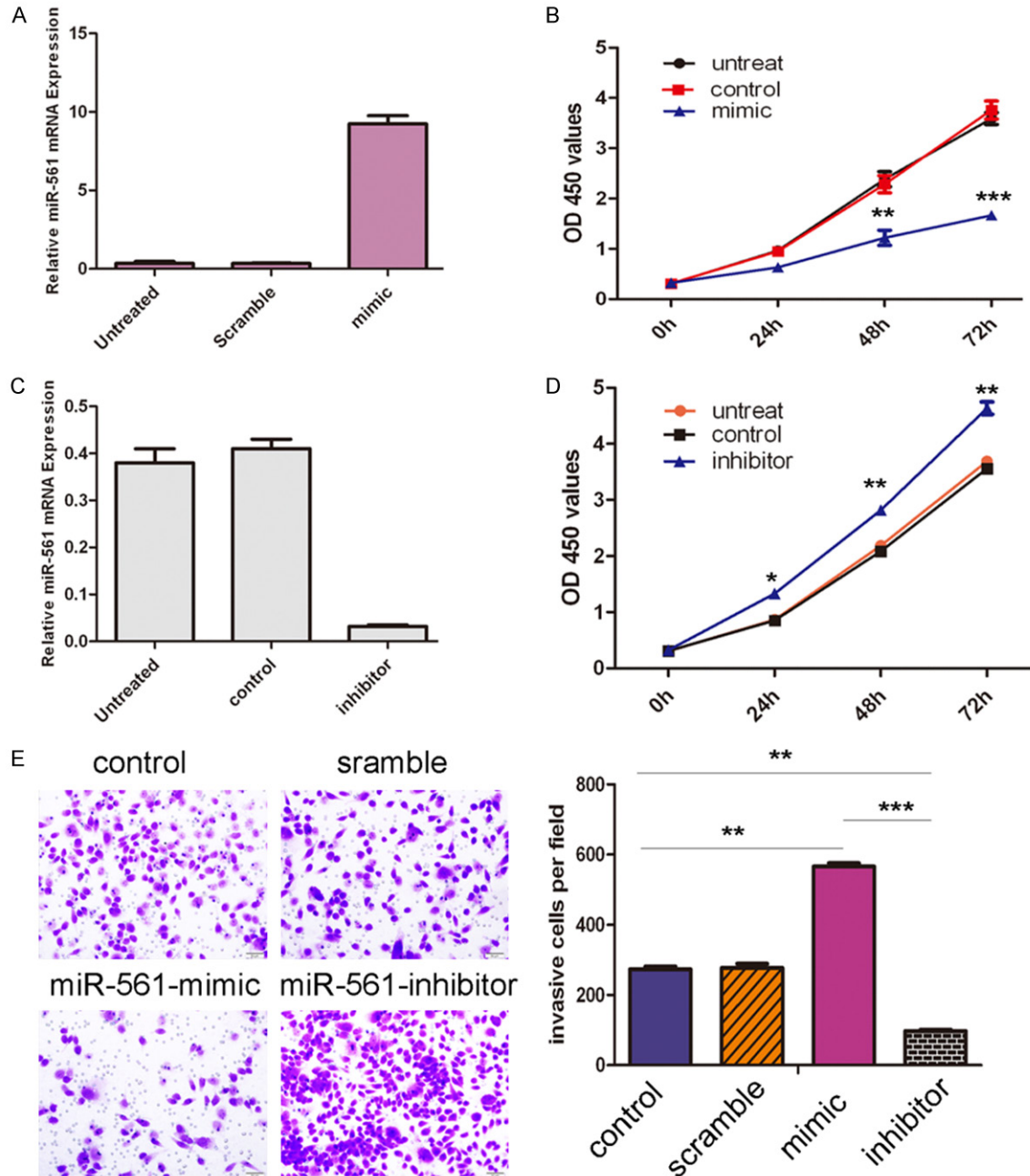
Figure 3. miR-561 inhibited GC cell proliferation and invasion. A. Expression levels of miR-561 were examined by real-time PCR after transfection of miR-561 mimics or scramble or no transfection. B. Growth of HGC-27 cells and MGC-803 cells were shown after transfection with miR-561 mimics or sramble or no transfection. The growth index as assessed at 0, 24, 48 and 72 h. C. Expression levels of miR-561 were examined by real-time PCR after transfection of miR-561 inhibitor or control or no transfection. D. Growth of HGC-27 cells and MGC-803 cells were shown after transfection with miR-561 inhibitor or control or no transfection. The growth index as assessed at 0, 24, 48 and 72 h. E. Transwell analysis of HGC-27 cells and MGC-803 cells after treatment with miR-561 mimics, inhibitors or scramble or control; the relative ratio of invasive cells per field is shown below, *p<0.05, **p<0.01, and ***p<0.001.

and invasion inhibition imposed by miR-561 (**Figure 5B** and **5C**). In summary, these data indicate that inhibition of c-Myc by miR-561 is responsible, at least in part, for the miR-561 inhibition of cell proliferation and invasion in human GC.
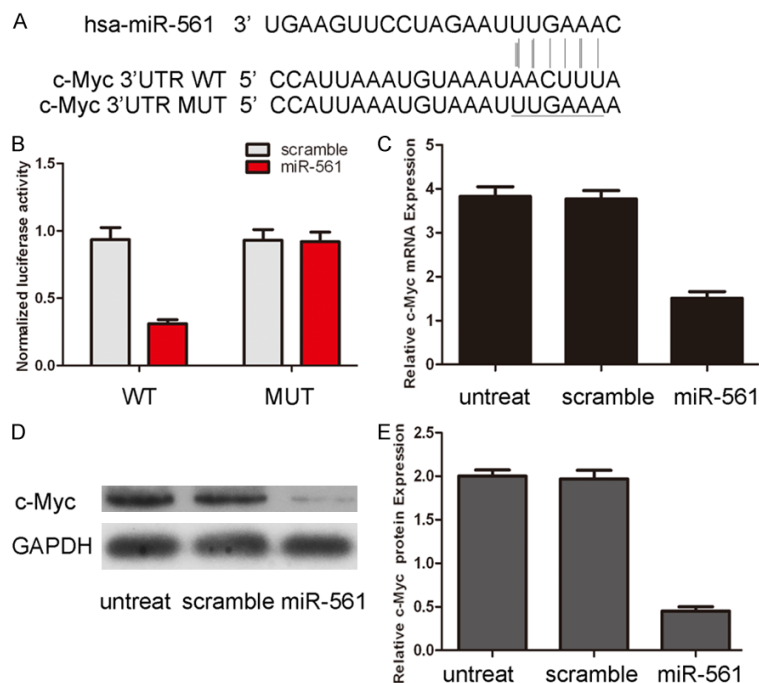
Figure 4. miR-561 downregulates c-Myc through interaction with its 3'UTR. A. The sequences of miR-561 binding sites within the human c-Myc 3'UTRs and schematic reporter constructs, in this panel, c-Myc-WT represent the reporter constructs containing the entire 3'UTR sequences of c-Myc. C-Myc-MUT represent the reporter constructs containing mutated nucleotides. B. The analysis of the relative luciferase activities of c-Myc-WT, c-Myc-MUT. The error bars are derived from triplicate experiments. C. qRT-PCR analysis of c-Myc mRNA expression in HGC-27 cells after treatment with miRNA mimics or scramble or no transfection. The expression of c-Myc was normalized to GAPDH. D. Western blot analysis of c-Myc expression in HGC-27 cells transfected with miR-561 mimics or scramble or no transfection. GAPDH was also detected as a loading control. E. The relative expression of c-Myc was shown.

C-Myc was inversely expressed with miR-561 in GC

As shown in **Figure 6A** and **6B**, the mRNA and protein expression of c-Myc was much higher in 4 GC cell lines than that of gastric epithelial-1 cells. We also found that the expression of c-Myc in GC tissues was higher than in the corresponding adjacent normal tissues (**Figure 6C** and **6D**). The scatter plot and the Pearson correlation analysis further showed that miR-561 expression was negatively correlated with the level of c-Myc mRNA in the GC samples (**Figure 6E**). These data suggested that decreased miR-561 expression was related to increased c-Myc levels in most GC patients.

Discussion

The majority of cancer-related deaths are caused by metastasis [22]. Therefore, effective intervention targeting cancer metastasis might decrease the mortality of cancer patients. Increasing evidences suggest an important role of miRNAs in cancer metastasis, such as miR-10b, miR-181a and miR-409 [12, 23-25]. However, the role of miRNAs in GC metastasis is still largely unknown. In our study, miR-561 was frequently downregulated in human GC cell lines and tissues and the downregulated miR-561 was associated with advanced clinical stage and lymph node-metastasis. Further studies showed that overexpression of miR-561 inhibited GC cell proliferation and invasion in vitro. Furthermore, c-Myc was identified as a direct and functional target of miR-561. The data from this study suggested that miR-561 acted as a novel tumor suppressor in GC and that downregulated miR-561 contributed to lymph node-metastasis and tumor progression in GC patients.

Deregulation of miR-561 is a frequent event in various cancers, suggesting that miR-561 may play an important role in tumor progression [19, 20]. Yu and his colleagues showed that miR-561 was down-regulated human colon cancer stem cells [21]. Previous study also showed that DAX-1 was a target of miR-561, which modulated acetaminophen-induced hepatotoxicity through DAX-1-mediated modulation of NR transactivation, indicating that miR-561 may represent a novel therapeutic target to manage acetaminophen overdose poisoning [26]. However, the expression and function of miR-561 in GC development remainuninvestigated. Our results showed that miR-561 was down-regulated in 44 (44/50, 88%) GC tissues compared with the adjacent tissues and the expression of miR-561 in GC tissues was lower than in adjacent tissues. We also found that lower expression of miR-561 in GC specimens was correlated with pTNM stage. Forced expression of miR-561 inhibited cell proliferationand invasion in GC
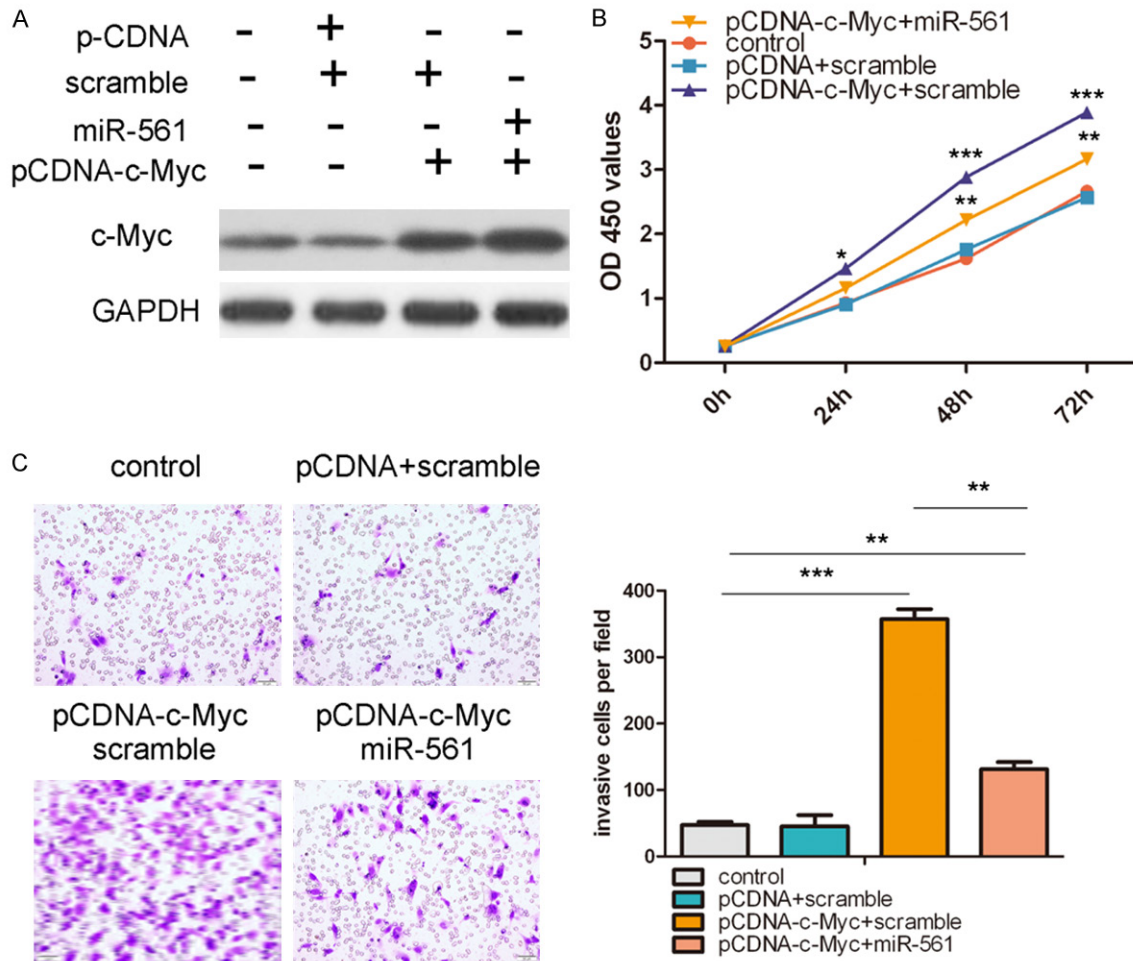
Figure 5. Overexpression of c-Myc partially rescued miR-561-inhibited cell proliferation and invasion. A. Western blot analysis of c-Myc in HGC-27 cells co-transfected with either miR-561 mimic or scramble and 2.0 µg pCDNA-c-Myc or pCDNA empty vector. GAPDH was also detected as a loading control. B. Cell growth curves in HGC-27 cells transfected with different combinations at 0, 24, 48 and 72 h. C. Transwell analysis of HGC-27 cells treated with different combinations. The relative ratio of invasive cells per field is shown right. *p<0.05, **p<0.01, ***p<0.001.

cell lines, indicating that repression of miR-561 might promote tumor progression in gastric carcinogenesis. All of this evidence indicated that miR-561 might contribute toGC development. miRNAdysregulation might result from various molecular mechanisms,, such as genetic mutation, epigenetic aberration and deregulated transcriptional activity [27, 28]. Among them, epigenetic aberration may play critical roles in the transcriptional silencing of tumor suppressor genes or suppressor miRNAs by specific DNA methylation and histone modification in cancers [29]. Therefore, further experiments, such as DNA methylation and histone modification assays, might be necessary to pinpoint the molecular mechanisms of miR-561 dysregulation in GC.

We next try to identify the molecular mechanism by which miR-561 acts as a metastasis suppressor in GC. Luciferase assay and Western blot analysis showed that miR-561 inhibited the expression of c-Myc by directly targeting its 3'UTR. C-Myc is a key basic helix-loop-helix leucine zipper transcription factor that acts as an important regulator of several cellular processes, including cell migration, growth and proliferation in various cells [30-32]. It is overexpressed in a number of human cancers, and its overexpression contributes to malignant transformation by regulating the expression of a number of genes participating in multiple aspects of tumorigenesis, such as angiogenesis, cell cycle progression, cell invasion, migration, metastasis, and angiogenesis
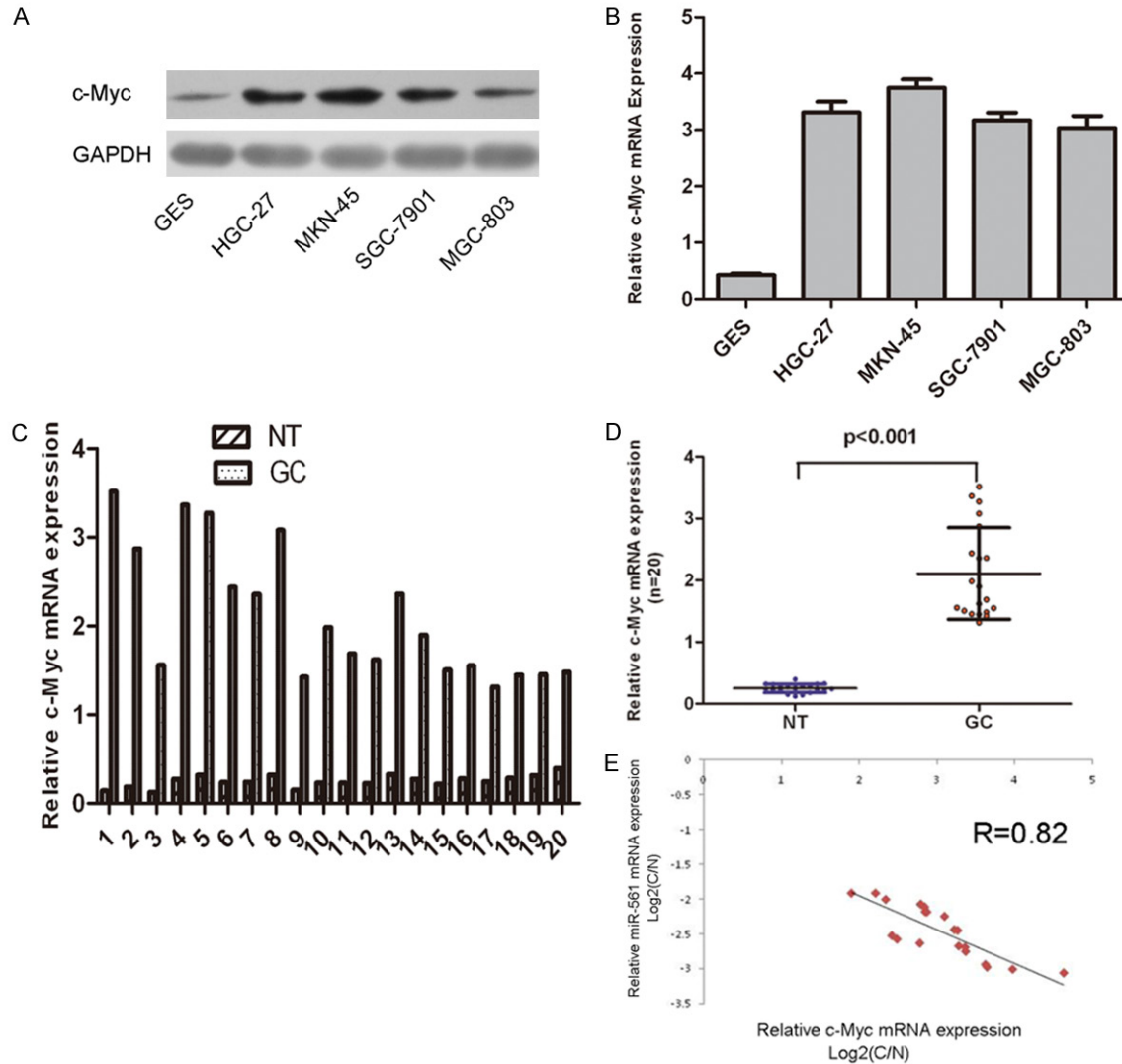
Figure 6. c-Myc was inversely expressed with miR-561 in GC. A. The relative c-Myc mRNA expression levels were determined by qRT-PCR in GC cell lines (SGC-7901, HGC-27, MKN-45, and MGC-803) and one normal gastric epithelial-1 cells (GES). B. Western blot analysis of c-Myc expression in GC cell lines (SGC-7901, HGC-27, MKN-45 and MGC-803) and one normal gastric epithelial-1 cells (GES). GAPDH was also detected as a loading control. C. qRT-PCR analysis of c-Myc expression in 20 pairs GC tissues and their corresponding adjacent normal gastric tissues. The expression of c-Myc was normalized to GAPDH. D. The expression of c-Myc in GC tissues was significant higher than in the corresponding adjacent normal tissues (NT). E. Analysis of correlation of miR-561 and c-Myc expression in GC tissues. (Two-tailed Pearson's correlation analysis, r = -0.82; p<0.01). Data was presented as log2 of fold change of GC tissues relative to non-tumor adjacent tissues.

[33-35]. Recent studies have shown that c-Myc has important roles in GC development and progression [36]. Its high expression is correlated with advanced disease stage, lymph-node metastasis and poor survival rates [37, 38]. Knockdown of c-Myc inhibited the invasion and metastasis of GC cell lines in vitro [39]. Consistent with that, we found that c-Myc was enriched in the primary GC tissues andinversely correlated to miR-561 levels. However, further studies with large number of primary GC samples were needed to confirm this interaction. These results demonstrate that miR-561 may act as a metastasis suppressor in gastric cancer by targeting c-Myc. Nevertheless, it should be noticed thatbesides c-Myc, lots of other genes could be regulated by miR-561. A more comprehensive profiling of gene dysregu-

lation by microarray is needed to enhance the selection of target gene candidates for further functional analysis.

In conclusion, our study show that miR-561 is downregulated in GC cell lines and that low expression of miR-561 is associated with lymph-node metastasis, poor pTNM stage. Moreover, enforced expression of miR-561 suppressed GC cell proliferation and invasion through directly targeting c-Myc. miR-561 may act as an important tumor suppressor in GC.

Disclosure of conflict of interest

None.

Address correspondence to: Huanwen Chen, Department of Cardiothoracic Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China. E-mail: lgr21d@163.com

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Table S1. Clinicopathologic charateristics of patients with GC

Parameter	Total samples	Percentage	miR-561 expression		P
			Low (n%)	High (n%)	
Age (years)					0.61
≥60	32	64%	28	4	
<60	18	36%	16	2	
Gender					0.73
Male	34	68%	30	4	
Female	16	32%	14	2	
Clinical TNM stage					
I	10	20%	6	4	
II	18	36%	16	2	
III	15	30%	15	0	
IV	7	14%	7	0	
Location					0.66
Proximal	22	44%	20	2	
Body	28	56%	24	4	
Metastasis					0.001
Present	7	14%	7	0	
Absent	43	86%	37	6	
Histological					
Diffuse	23	46%	21	2	0.69
Intestinal	27	54%	23	4	

Table S2. Primer sequence

Name	Sequence (5'-3')
miRNA reverse transcription prime	
miRNA-561	GTATCCAGGTCTGCAGGGTCCGAGGTATACTGGATCGCTACGACTCACAT
U6 snRNA	AAAATATGGAACGCTTCACGAATTTG
Real-time PCR primer sequence	
miRNA-561	CAAAGUUUAAGAUCCUUGAAGU
	TCAACTGGTGTCGTGGAGTCGGC
U6 snRNA	CTCGCTTCGGCAGCACATATACT
	ACGCTTCACGAATTTGCGTGTC
GAPDH	AATGGGCAGCCGTTAGGAAA
	TGAAGGGGTCATTGATGGCA
c-Myc	TGCTAAGAAGATTGGTGCTGTA
	GCGAAGGGCTGAGACATTTAC